Pectin-honey hydrogel: Characterization, antimicrobial activity and biocompatibility

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Abstract

Background: Novel pectin-honey hydrogels have been developed and characterized as medical device. Ideally, a wound dressing should maintain optimal fluid affinity, permit moisture evaporation, protect the wound from microbes, and have shape-conformability, biocompatibility, and antibacterial activity. **Objective:** A novel, simple and fast method to produce pectin-honey wound dressings is described. **Methods:** The properties of these pectin-honey hydrogels were investigated, including swelling ability, water vapour transmission rate, hydrogen peroxide production, methylglyoxal content and antibacterial activity. Biocompatibility was assessed by proliferation assays using cultured fibroblast cells and by *in vivo* study with subcutaneous and intraperitoneal implantation in rats. **Results:** Hydrogel showed a good water vapour transmission rate, fluid uptake and were not cytotoxic for fibroblasts. The hydrogel demonstrated good antibacterial activity toward clinically relevant pathogens, including *S. aureus* and *E. coli*. Biocompatibility was confirmed by the measurement of plasma levels of interleukin (IL)1 beta, IL-6, tumour necrosis factor (TNF) alpha, and prostaglandin (PG)E2. No histological changes were observed. **Conclusions:** The presence of a natural active component, conformability, and complete resorbability are the main characteristics of this new biocompatible biomaterial that is well tolerated by the body, possibly improves healing, may be used for surgical complications prevention, with a simple and inexpensive production process.

Keywords: biocompatibility, biomaterial, pectin-honey hydrogels, wound dressing, medical device

Introduction

Healing wounds are sites that are easily attacked by bacteria, which leads to the formation of a biofilm that devitalises tissues [1]. The development of a new wound dressing is a focus area for many researchers since the dressing can create a barrier against infection while maintaining a physiological environment in contact with the wound [2-3]. These dressings help to maintain a moist environment at the wound site, promote tissue healing, and reduce infection, pain, and costs [1, 4-5]. Antimicrobial agents play an important role in reducing bacterial contamination, but the resistance of pathogens to these substances has led to a decrease in the efficacy of antibiotics. Researchers have therefore advised against the use of systemic antimicrobial agents for the treatment of wounds [1, 6].

Various wound dressings consisting of vegetable fibres, protective films, hydrogels, and hydrogel enriched with nitrogen oxides are available commercially [1-2, 5, 7]. Hydrogels are reported to be suitable for use in healing wounds but they have limitations in current use, which include a requirement for high frequency of application, inactivation by wound fluids, and formation of eschars [5]. The requirement for a new antimicrobial substance led to revaluation of ancient remedies, including the use of honey [8-9].

Honey has been used alone or in combination with other compounds for medical use since ancient times. Honey is a heterogeneous substance, has antimicrobial activity, anti-inflammatory effects and increase the healing process following skin or peritoneal damage [8, 10]. It contains high levels of glycine, methionine and proline which are all fundamental for collagen formation and fibroblast deposition, which are the main factors for wound healing [11].

Manuka honey, produced in New Zealand, is the most studied honey having antibacterial properties against major aerobic and anaerobic bacteria species [12-13].

Several components are known to contribute antibacterial activity of honey. The osmotic effect of the sugars in honey and its characteristically acidic pH are known factors hindering bacterial multiplication. Recently, two components of honey, hydrogen peroxide (H2O2) and methylglyoxal (MGO), have been identified as effective antibacterials [14-16].

An ideal wound dressing is yet to be developed. Ideally, a wound dressing should maintain optimal fluid affinity, permit moisture evaporation, protect the wound from foreign microbes, and have shape-conformability, biocompatibility, and antibacterial activity [2,4-5,17].

Based on these premises, the aim of the present study was to describe and characterize the properties of pectin-honey hydrogels (PHHs) for wound healing and to assess their biocompatibility through an in vitro and in vivo assay.

Material and methods

Materials

Honey was purchased from Manuka Health (66 Weona Court,Te Awamutu 3800, New Zealand) and pectin from Ardet s.r.l. (Torino, Italy). Culture media, that is, tryptone soy agar (TSA), tryptone soy broth (TSB), peptone water, and 5% sheep blood agar, were purchased from Oxoid (Milan, Italy). Escherichia coli (Turin strain) and Staphylococcus aureus (Turin strain) isolated from canine wounds were used for this work.

Preparation of PHHs

The preparation method was modified after the procedure described by Walker. (Walker, 1942) [18] Briefly, the pectin-honey hydrogels were prepared starting from a solution (1:1 v/v) of liquid honey (Manuka Health, New Zealand) and sterile deionized water. The same volume of pectin powder (ARDET s.r.l., Italy) was then added and with continuous stirring until the mixture was homogeneous. The resulting gel was spread into 2 mm thick films and hot air dried at 40+/- 0.5 °C for 6 h. Then it was cut into squares of 5 x 5 cm and further conditioned in an air drier at 25+/-

96 1 °C for 5 days. The films were then collected and packed in polyethylene under vacuum
97 conditions. All membranes were sterilized by gamma-irradiation at 25 KGray at the Sterigenics
98 Italy (Sterigenics Italy, Minerbio (BO), Italy) [19].

99 ***Fluid uptake test (Swelling test)***

100 To investigate the fluid swelling ratio of PHHs, samples were cut into disks with a diameter of
101 approximately 25 mm. The dry weights (Wdry) of the membranes were measured and recorded.
102 Afterwards, pre-weighed dry samples were immersed in PBS solution, pH 7.4, at 37°C. The
103 weights of the swollen PHHs were determined every 5 min subsequently by sandwiching the
104 membranes between two paper towels to remove excess water on the surface, and then wet
105 weights (Wwet) were measured. All experiments were performed in triplicate. The swelling
106 ratios were calculated as the average value according to the following formula:

107 $DS = [(Ww – Wd)/Wd] \times 100$

108 Where Ww and Wd represent the weights of wet and dry samples, respectively.

109 ***Water vapour transmission rate (WVTR)***

110 The moisture permeability of the PHH was determined by measuring the WVTR. A piece of the
111 specimen was fixed over the top of a tube (diameter, 34 mm) containing 10 mL PBS. The tube
112 was then placed in an incubator at 37°C and 35% relative humidity. The membranes were
113 weighed at regular intervals of time and the weight loss was recorded and plotted on a graph
114 versus time. The WVTR was calculated from the slope of the graph by the following formula:

115 $WVTR\ g\ (m2/day) = (slope \times 24)/A$

116 Where A is area of the sample (m2). Experiments were performed in triplicate.

117 ***H2O2 analysis***

118 The analysis of H_2O_2 from honey and PHHs was carried out according to the method reported by
119 Long (1999) [20] (ferrous ion oxidation-xylenol orange [FOX] assay), with minor modifications.

The stock reagents were as follows: reagent 1, 4.4 mM butylated hydroxy toluene (BHT) in HPLC-grade methanol; reagent 2, 1 mM xylenol orange and 2.56 mM ammonium ferrous sulphate in 250 mM H_2SO_4; working reagent: one volume of reagent 2 added to nine volumes of reagent 1. Approximately 200 mg of honey were diluted with deionized water to the ratio 20% w/w and immediately kept in a thermostat at T = 37 °C, and 100-µL aliquots were added to 900 µl of FOX working reagent. After 20 min of incubation at room temperature, absorbance was measured at λ= 560 nm against a blank comprising 900 µL of working reagent and 100- µL aliquots of distilled water (absorbance of diluted honey was negligible at the operative wavelength). H_2O_2 was monitored at 5, 10, 15, 20, 30, 60, and 120 min and up to 24 and 48 h of incubation.

To reduce the processing time required to extract the water-soluble components from PHHs, their size was reduced prior to incubation by cutting the specimens into small pieces with the aid of a small and clean knife. Aliquots of 100 µL of the aqueous layer of the final mixture (40% w/w) were subjected to FOX analysis as described above for honey. Monitoring of H2O2 was carried out at 60 min, 90 min, 24 h, and 48 h. The FOX assay was calibrated using standard H2O2 (molar extinction coefficient, 43 M-1 cm-1; absorbance wave length of H2O2 λ = 240 nm; linear range, 0–50 µM). Ten units of catalase were sufficient to destroy all the H_2O_2 immediately. Controls with catalase were used to exclude interference due to honey constituents.

Methylglyoxal (MGO) analysis

MGO was evaluated by the method proposed by Wild (2012) [21] with slight modifications. The method is based on the reaction between N-acetyl-L-cysteine (Sigma Aldrich) and MGO at room temperature. Samples were diluted in water (340 mg/ml) and the reaction was performed in 100 mM sodium dihydrogen phosphate buffer (adjusted to pH 7.0 with 10 M NaOH) at 22 °C. For the standard curve of the reaction, different concentrations of MGO (0.5, 1, 2, and 5 mM) were used.

MGO solutions (Sigma Aldrich) equivalent to 0.5, 2, and 5 mM and 10 µl of each honey solution (170 mg HBM/ml water) were added up to a volume of 980 µL with sodium dihydrogen phosphate. The reaction was started by adding 20 µL 500 mM N-acetyl-L-cysteine, and the absorption was recorded after 7 min. The condensation product, N-α-acetyl-S-(1-hydroxy-2-oxo-prop-1-yl) cysteine, was determined by recording the absorption at 288 nm (UVIKON 923, Bio-Tek Instrument). Results are given in µmol/mg of honey.

Protein content analysis of honeys

Honey samples were diluted in water and the protein content was determined using the BCA protein assay kit according to the manufacturer's instructions (Pierce, BCA Protein Assay).

Microbiological analysis

The success of sterilization was verified by the absence of bacterial growth on solid medium (5% sheep blood agar) at 37 °C for 24 h, both in aerobiosis and anaerobiosis. Subsequently, the antibacterial activity of PHHs was determined against S. aureus and E. coli, which had been previously isolated from canine wound infections. The agar good diffusion method was used to screen the antimicrobial activity of the Manuka PHHs [22]. Clinical strains were grown overnight in TSB at 37°C and adjusted to 0.5 McFarland standard. Each culture was inoculated on the surface of Petri plates. Subsequently, wells with 6-mm diameter were bored into the surface of the agar. The wells were filled with 6 mm of a Manuka honey-based patch, 80 µl of a Manuka honey sample 100% v/v (as positive control), 80 µl of a Manuka honey sample 50% v/v (as a positive control that resembles the concentration of honey present in PHHs), and 6 mm of pectin (as negative control). Plates were incubated at 37°C, and after 24 h, the diameters of the inhibition zones were measured. Each assay was carried out in triplicate.

In vitro cytotoxicity assay

The cytocompatibility of PHHs was evaluated using L929 cells (mice fibroblasts) (ECACC Cell Lines-Sigma Aldrich, Milan, Italy) that were cultured in 75 mL flasks containing Modified Eagles Medium (MEM; Sigma Aldrich, Milan, Italy), 10% fetal bovine serum (FBS; Sigma Aldrich, Milan, Italy), 2% L-glutamine (Sigma Aldrich, Milan, Italy), and 2% penicillin-streptomicin-amphotericin B solution (Sigma Aldrich) at 37°C with 5% CO2, 95% air, and complete humidity. When a confluence of 80% was reached, cells were detached using 0.1% trypsin/EDTA solution (Sigma Aldrich, Milan, Italy), centrifuged, and counted using Trypan Blue solution (Sigma Aldrich) with a Burker chamber. Cells were either resuspended at a concentration of 5×105 cells/mL or stored at -80°C for further analysis. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay, a routine method for quantifying cell viability, was used [23].

Cells (5×10^5 cells/100 µL) were transferred into 96-well plates in order to perform the MTT assay at 24, 48, and 72 hours after seeding. After an overnight incubation at 37°C and 5% CO2, the medium was changed (MEM, FBS 10%, antibiotic and antimycotic solution 2%, L-glutamine 2%), and serial decreasing concentrations of honey membranes dissolved in phosphate buffered saline (PBS) solution, starting from 0.1 g/ml, were added. After adding MTT (Sigma Aldrich, Milan, Italy) solution (4 mg/ml in PBS), the plates were incubated at 37 °C and 5% CO2 for 4 hours. At the end of incubation, the medium was removed, and 100 µL of dimethyl sulfoxide (DMSO; Sigma Aldrich, Milan, Italy) was added in order to lysate the formazan crystals. The plates were further incubated for 10 minutes on a continuous shaker at room temperature, and the optical density of the wells was determined using a plate reader (Poverwave x; Bio-Tek Instruments Inc., Winooski, USA) at a wavelength of 570 nm.

In vivo biocompatibility study

All procedures were approved by the Bioethical Committee of the University of Turin and by the Italian Ministry of Health (protocol number 304/2015-PR, 20/04/2015).

A total of 39 adult male Sprague-Dawley rats, weighing 225-250 grams, were purchased by Harlan Laboratories (Italy). All rats were housed in single cages for 7 days prior to the beginning of the experiment. The room temperature was set at 23°C throughout the duration of the experiment and cages were cleaned daily. Animals were fed with a commercial diet and water was given ad libitum. Anaesthesia was induced by administering 5 mg/kg of xylazine (Rompum®, Bayer Animal Health, Italy) and 50 mg/kg of tiletamine and zolazepam (Zoletil 50, Virbac, Italy) intramuscularly. Anaesthesia lasted for approximately 1 hour. Under anaesthesia, blood samples were collected from the caudal vein to perform biochemical analysis. Soon after, the ventral hair was shaved and the skin was prepared using a 3-step iodopovidone – chlorhexidine scrub. The animals were randomly assigned to the treatment or control groups. A 4-cm midline incision was made in the abdominal wall. In the treatment group, subcutaneous and intraperitoneal implantation of PHH was performed as previously described [24]. Briefly, one square each of PHH measuring 1 × 1 cm was implanted intraperitoneally under the left abdominal wall and subcutaneously at ~1 cm left to the midline between the muscle and skin. In the control group, only the surgical procedure was performed without PHH implantation. In both groups, the midline incision was closed in two layers, with 3-0 USP glycomer 631 for the fascia, and with 3-0 USP nylon for the skin. Each surgical procedure lasted about ~20 minutes.

Blood samples for the biochemical analysis were collected from the caudal vein. The blood was collected into tubes before surgery and at 6 h (T6), 24 h (T24), and 72 h (T72) post-surgery and at the time of euthanasia. Plasma levels of IL-1β, IL-6, TNF-α and PG(E2) were measured by using a commercial ELISA kit (Rat IL1 beta ELISA kit, Booster Biological Technology; Rat Il-6

ELISA, AB Frontier; Rat TNF alpha ELISA, AB Frontier; Prostaglandin E2 Express EIA kit, Cayman Chemical).

At 0, 6, 24, 72 hour's post-surgery, three rats from each group were euthanized and target organs (liver, kidney, left abdominal wall) were collected and fixed in 4% formaldehyde. These tissues were sectioned, stained in H&E, and observed by two pathologists in a blinded manner [24].

Statistical analysis

Normality of the data was evaluated using the Shapiro–Wilk normality test. For the in vitro cytotoxicity assay, all experiments were performed in triplicate, and the data are representative of at least three independent experiments. The results, expressed as mean ± SEM values, were analysed using the Kruskal-Wallis test and a Dunn's post-test.

For plasma cytokine levels, all experiments were performed in duplicate. For IL-1β, IL-6, and TNF-α, the results have been expressed as median (95% IC) values and were analysed using the one-way ANOVA test. For PG(E2), the results are expressed as median (95% IC) and were analysed using the Friedman test. Statistical analysis was performed with the GraphPad Prism 6.01 software. Values with $p < 0.05$ were considered significant.

Results

Fluid uptake test (swelling test)

The weight of PHHs immersed in PBS solution under physiological mimicking conditions (pH 7.4, 37°C). The fluid content increased to about 150% after 180 minutes. The results from the fluid uptake experiment revealed that the dressing has wide capacity to prevent fluid accumulation if used on wound.

WVTR

The transmission of water vapour through the membranes is an important parameter for the evaluation of their effectiveness as a hydration factor when placed on a wound. The WVTR

recommended for wound dressing is 2000–2500 g/m2/day in order to ensure proper wound moisture without risk of dehydration or excessive production of exudates [1,4]. A good WVTR facilitates the healing process because it improves cell migration and promotes re-epithelialization.

The water loss from a fully hydrated dressing on exposure to air was evaluated. The mean evaporative water loss from PHHs was 2689.8 ± 158.5 g/m2/day.

H2O2-producing activity

All the honey samples, before inclusion in the membranes, were able to produce significant amounts of H_2O_2 while no H_2O_2 development was observed in the case of corresponding PHHs (data not shown). In Manuka honey, at shorter incubation times up to 90 min of incubation, the concentration of H_2O_2 generated by honey glucose oxidase was in the range reported in the literature for other types of honey with different methods of analyses (1–2 mM at 30 min of incubation) [16, 25-27]. By contrast, Manuka honey showed the lowest H_2O_2production at all incubation times, probably because of its high content of MGO, which has previously been suggested to be a glucose oxidase-inhibitory agent [16, 25]. At longer incubation times (24 and 48 h), the tested honey samples displayed a significantly different behaviour: The dramatic loss of H_2O_2producing activity found for PHHs indicated that their production procedure, which included a heating step at 80 °C and exposure to γ-rays for final sterilization, induced complete loss of glucose oxidase activity. This demonstrated that the preserved antibacterial activity in the microbiological testing was generated solely by the action of nonperoxide agents.

MGO analysis

Dihydroxyacetone (DHA) is a direct precursor of MG in Manuka honey [15]. The MGO concentration in PHHs, determined by the N-acetyl-L-cysteine assay, was 0.26 ± 0.07 μmol/mg of proteins. The PHHs had higher MG concentration than bulk honey. MGO content is important

because it can serve as a suitable quality and cost parameter for Manuka honey. The H_2O_2 and MG content is responsible for the antibacterial activity of honey [28] and PHHs maintain antibacterial activity similar to that of bulk honey.

Microbiological analysis

Manuka membranes did not show bacterial contamination after sterilization by gamma-irradiation. Table 1 outlines the antibacterial activity based on the clear zone that was produced.

In vitro cytotoxicity assay

Results concerning the effects induced by different concentrations of dissolved honey membrane on viability of L929 cells are represented in Figure 1.

In vivo biocompatibility study

Three rats from the treated group died of ascites in the first 24 hours. The gross evaluation in 36 rats showed no wound site infection or presence of adhesions. On performing histological analysis 24 and 72 hours' post-surgery, the tissue near the implant was found to be characterized by the presence of fibroblasts with some cellular response, including lymphocytes, macrophages, and neovascularisation. No reaction was observed in distant organs. Thus, the PHHs did not induce a foreign body reaction. The differences in the blood levels of the IL-1β, IL-6, TNF-α and PG(E2) at the 0, 6, 24, and 72 hours' time points were not statistically significant. The results are summarized in Table 2.

Discussion

The new membranes may be used as wound dressings as they have a good WVTR and fluid uptake and show no cytotoxicity to fibroblasts; they also have good swelling capability, which is an important factor for reducing the risk of wound dehydration.

285 The results obtained by the citoxicity assay after 24 hours from the seeding, in presence of
286 decreasing concentrations of dissolved honey membranes, have shown a statistically significant
287 decrease ($p<0.05$) of the cells treated with the highest concentration (0.1 g/ml) compared to the
288 control ones was observed. At 48 hours a trend in cell proliferation was found: highest
289 concentrations seemed to induce a inhibition in cell growth while decreasing the concentration,
290 the cell proliferation seemed to increase, even if it was not possible to highlight significant
291 differences. Also at this time point it was possible to appreciate a statistically significant
292 decreasing ($p<0.05$) of the cells treated with the highest concentration (0.1 g/ml) compared to the
293 control.
294 After 72 hours of incubation, the highest concentrations (from 1:2 to 1:64) caused an inhibition in
295 cell growth while the lowest (1:256 and 1:512) induces a statistically significant increase in cell
296 growth compared to the control. During the *in vivo* experiments, tree rats died: in authors'
297 opinion, this was because of the excessively large sheet of membrane implanted intraperitoneally
298 because, initially, a dimension of 2 × 2 cm was chosen. Reducing the dimension of the implanted
299 PHHs to 1 × 1 cm did not cause any intraperitoneal accumulation of fluid, as determined from
300 macroscopic examination after euthanasia of the remaining rats. The tissue response to
301 intraperitoneal and subcutaneous implants showed a similar macroscopic and histological pattern.
302 In the light of PHHs antibacterial activity [12] and since administration of systemic antibiotics
303 does not always lead to good outcomes in terms of: wound healing, matrix penetration of the EPS
304 biofilm and antibiotic resistance, in this study we propose the use of Manuka honey to prepare
305 PHH for wound dressings. Interestingly, our membranes demonstrate a good antibacterial activity
306 toward clinically relevant pathogenic microorganisms such as S. aureus and E. coli.
307 Honey membranes possess a wide variety of properties that can make them suitable (as for other
308 natural materials such as chitosan hydrogels) [2, 24, 29], for very different uses that we can

hypothesise ranging from wound healing to adhesion prevention to drug delivery. The presence of natural active components, conformability, and complete resorbability are the main characteristics of this new biocompatible biomaterial that respects the pathophysiology of tissue, is well tolerated by the body, possibly improves healing, and may be used for the prevention of surgical complications. Furthermore, the production of these devices is extremely simple and inexpensive.

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Table legend

Table 1: Mean zones of inhibition (diameter [including that of the well], 6 mm)

Table 2: Results for blood levels of the IL-1β, IL-6, TNF alpha and PG(E2)

Figure legend

401 Figure 1: MTT assay (N=8) to evaluate the modulation of L292 cells growth after the treatment
402 with decreasing concentrations of dissolved honey membrane in PBS at different experimental
403 time points (24, 48, and 72 hours).